ANNEX G

Oncogene (2003) 00, 1–7

www.nature.com/onc

ORIGINAL PAPER

An agonist to the A_3 adenosine receptor inhibits colon carcinoma growth in mice via modulation of GSK-3β and NF-κB

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A_3 adenosine receptor (A_3AR) activation with the specific agonist CF101 has been shown to inhibit the development of colon carcinoma growth in syngeneic and xenograft murine models. In the present study, we looked into the effect of CF101 on the molecular mechanisms involved in the inhibition of HCT-116 colon carcinoma in mice. In tumor lesions derived from CF101-treated mice, a decrease in the expression level of protein kinase A (PKA) and an increase in glycogen synthase kinase-3β (GSK-3β) was observed. This gave rise to downregulation of β-catenin and its transcriptional gene products cyclin D1 and c-Myc. Further mechanistic studies *in vitro* revealed that these responses were counteracted by the selective A_3AR antagonist MRS 1523 and by the GSK-3β inhibitors lithium and SB216763, confirming that the observed effects were A_3AR and GSK-3β mediated. CF101 downregulated PKB/Akt expression level, resulting in a decrease in the level and DNA-binding capacity of NF-κB, both *in vivo* and *in vitro*. Furthermore, the PKA and PKB/Akt inhibitors H89 and Worthmannin mimicked the effect of CF101, supporting their involvement in mediating the response to the agonist. This is the first demonstration that A_3AR activation induces colon carcinoma growth inhibition via the modulation of the key proteins GSK-3β and NF-κB.

Oncogene (2003) **0**, 000–000. doi:10.1038/sj.onc.1207355

Keywords: A_3 adenosine receptor; CF101; colon carcinoma; β-catenin; GSK-3β

Introduction

The A_3AR is a G_i-protein-coupled receptor containing seven α helical spanning membrane domains. A_3AR was found to be expressed in different tumor cell lines, including Jurkat T, pineal gland, astrocytoma, melanoma as well as colon and prostate carcinoma (Gessi *et al.*, 2001; Merighi *et al.*, 2001; Suh *et al.*, 2001; Trincavelli *et al.*, 2002a; Fishman *et al.*, 2003; Madi *et al.*, 2003; Ohana *et al.*, 2003). A_3AR activation leads to inhibition of adenylyl cyclase activity, cAMP formation and PKA expression, resulting in the initiation of various signaling pathways which may include the MAPK and the PI3K (Poulsen and Quinn, 1998; Olah and Stiles, 2000; Trincavelli *et al.*, 2002b).

Our earlier studies demonstrated that melanoma cells highly express A_3AR, and suggested that it may serve as a target for tumor growth inhibition. A_3AR activation by the synthetic agonist 1-deoxy-1-[6-[[(3-iodophenyl)-methyl]amino]-9*H*-purine-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA) inhibited the growth of melanoma both *in vitro* and *in vivo* (Fishman *et al.*, 2001, 2002a, b, 2003; Ohana *et al.*, 2001; Madi *et al.*, 2003). The mechanistic pathway involved downregulation of the Wnt signaling pathway. It was found that IB-MECA inhibited the expression of PKAc and PKB/Akt, thereby preventing the phosphorylation and inactivation of GSK-3β. Consequently, GSK-3β was shown to phosphorylate β-catenin and prevent its translocation to the nucleus, resulting in downregulation of cyclin D1 and c-Myc (Fishman *et al.*, 2002b; Madi *et al.*, 2003). PKB/Akt is also known to control NF-κB level by phosphorylating downstream proteins, which in turn release NF-κB from its complex (Madrid *et al.*, 2001). Similar to β-catenin, NF-κB translocates to the nucleus, where, among other genes, it induces the transcription of c-Myc and cyclin D1 (Joyce *et al.*, 2001).

Our previous studies showed that CF101 is efficacious in suppressing the growth of primary and liver metastasis of CT-26 colon carcinoma cells in syngeneic experimental tumor models in mice (Ohana *et al.*, 2003). In addition, CF101 inhibited the growth of subcutaneous HCT-116 human colon carcinoma cells in a xenograft model in mice.

Aberrant activation of Wnt signaling, caused by mutations in β-catenin or APC, is a critical event in the development of colorectal tumors. In these cases, GSK-3β fails to phosphorylate β-catenin, which accumulates in the cytoplasm. β-catenin then translocates to the nucleus where, in association with Lef/Tcf, it induces the transcription of cyclin D1 and c-Myc (Morin, 1999).

The present study is focused on the molecular mechanism involved in the inhibition of colon carcino-

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Received 31 July 2003; revised 5 November 2003; accepted 12 November 2003

ma growth by CF101. We explored the signaling modulation of GSK-3β and NF-κB, both of which are affected by PKB/Akt (which is downstream to PI3K) and are known to regulate the level of the important oncogenes cyclin D1 and c-Myc. A major role for GSK-3β in mediating these responses is discussed.

Results

CF101 inhibits colon carcinoma growth in vivo *and modulates the expression level of A_3AR and downstream cell growth-regulatory proteins in tumor lesions*

HCT-116 colon carcinoma cells were engrafted subcutaneously into nude mice. When tumor reached the size of 150–200 mm^3, the mice were treated daily orally with CF101. Tumor growth was suppressed in the CF101-treated group in comparison to the vehicle-treated group (Figure 1a). On the day of study termination, 52 + 6.1% ($P<0.001$) tumor growth inhibition was observed. To evaluate the effect of chronic CF101 treatment on A_3AR expression and downstream cell growth-regulatory proteins, extracts were prepared from tumor lesions and subjected to Western blot (WB) analysis. In the group of mice killed 2 h after the last treatment, the expression level of A_3AR, PKAc, β-catenin, NF-κB, c-Myc and cyclin-D1 was downregulated, whereas GSK-3β was upregulated. In the group of mice killed 16 h after the last treatment, A_3AR expression was similar to that of the vehicle-treated group. Interestingly, in this group, most of the cell growth-regulatory proteins were decreased in comparison to the control group, indicating that continuous downregulation is achieved upon chronic CF101 treatment. Taken together, these data show that receptor downregulation occurs shortly (2 h) after CF101 treatment, leading to modulation of downstream proteins, and that A_3AR was not desensitized despite chronic activation (over a 20-day period). The expression of the receptor returned to normal levels 16 h after CF101 administration, demonstrating that, even after chronic activation, the receptor is fully expressed (Figure 1b).

CF101 modulates the expression level of A_3AR and downstream cell growth-regulatory proteins in vitro

To further study the association between A_3AR activation and the expression of downstream cell growth-regulatory proteins, HCT-116 colon carcinoma cells were incubated in the presence of CF101 (10 nM) for 15 min. Proteins were extracted and analysed by WB. Similar effects of CF101 to those seen *in vivo* were recorded. The expression level of the two kinases PKAc and PKB/Akt was downregulated, while the expression of their downstream substrate GSK-3β was upregulated. The levels of the coactivator β-catenin and the downstream target genes cyclin D1 and c-Myc were decreased (Figure 2a). To confirm that these responses are mediated via the A_3AR, the antagonist MRS 1523 was introduced to the culture system. The antagonist

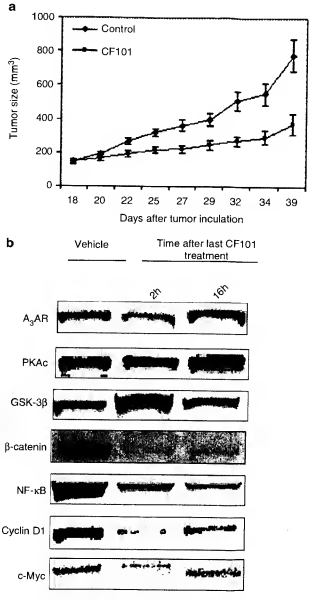
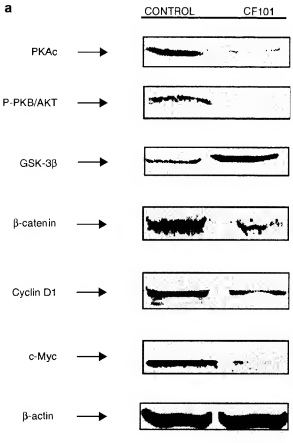


Figure 1 Inhibition of colon carcinoma cell growth in nude mice and modulation of cell growth-regulatory proteins in tumor lesions. HCT-116 cells were subcutaneously engrafted to nude mice. CF101 (10 μg/kg) treatment was initiated when tumor reached a size of 150 mm^3, and was given twice daily for 21 consecutive days. On day 21, the mice were killed 2 or 16 h after CF101 treatment. Tumor lesions were removed and protein extracts were prepared. (a) Tumor size was measured every 4 days. The curve represents a comparison between the vehicle and CF101-treated groups. (b) Immunoblots showing the effect of CF101 on cell growth-regulatory proteins derived from the colon carcinoma tumor lesions. A_3AR was downregulated 2 h after treatment and fully expressed after 16 h. Downstream cell growth-regulatory proteins were modulated upon CF101 treatment

counteracted the effect of CF101, thereby retaining the

control levels of PKAc, GSK-3β and cyclin D1, demonstrating the specificity of the response (Figure 2b). To further elucidate the role of PKA and PKB in mediating cell response to CF101, their activity was mimicked by H89 and Worthmannin (PKA and PKB/Akt inhibitors, respectively). Figure 2c depicts an increase in GSK-3β level upon treatment with the two inhibitors.



CF101 deregulates GSK-3β and downstream key signaling proteins

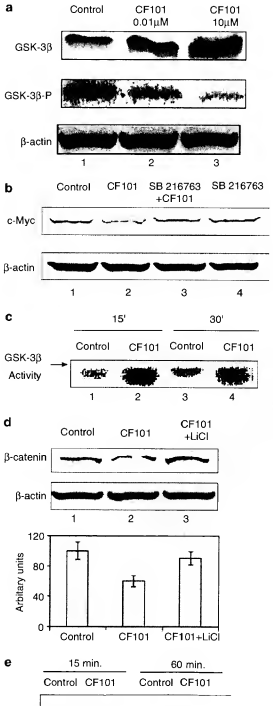
The next set of experiments was carried out to assure that CF101 decreased cyclin D1 and c-Myc levels via modulation of GSK-3β. We therefore compared the active nonphosphorylated GSK-3β level to its nonactive phosphorylated form. Consistent with the former data, we found that, upon CF101 treatment, the nonphosphorylated form was upregulated, whereas the phosphorylated one was decreased (Figure 3a). SB216763, an inhibitor to GSK-3β, counteracted the ability of CF101 to downregulate c-Myc, confirming that this response was GSK-3β mediated (Figure 3b). Furthermore, marked increase in the activity of GSK-3β was also noted after 15 and 30 min (Figure 3c). To assess whether the decrease in β-catenin is mediated via its phosphorylation by GSK-3β, HCT-116 cells were treated with lithium chloride that inhibits the serine/threonine phosphorylation activity of GSK-3β. Indeed, lithium treatment reversed the decrease in β-catenin expression level ($36 + 3.4\%$, $P < 0.002$), confirming that this response is GSK-3β mediated (Figure 3d). In addition, the nuclear level of LEF-1 in the CF101-treated cells was downregulated (Figure 3e), supporting the notion that less β-catenin was associated with LEF-1 and subsequently translocated to the nucleus.

Effect of CF101 on the level and transcription activity of NF-κB

Activated PKB/Akt can phosphorylate IκB kinase, leading to further phosphorylation events and the release of NF-κB from its complex with IκB. Accordingly, we examined whether the downregulation of PKB/Akt will affect the protein expression and DNA-binding capacity of NF-κB, also known to induce cyclin D1 and c-Myc transcription. Indeed, decreased NF-κB level was seen in protein extracts derived from CF101-treated HCT-116 cells (Figure 4a). This decrease was blocked when the antagonist MRS 1523 was present in the culture medium together with CF101, demonstrating the specificity of this response. Moreover, electrophore-

Figure 2 Modulation of cell growth-regulatory proteins in HCT-116 colon carcinoma cells upon CF101 treatment *in vitro*. (**a**) Immunoblots showing the effect of 10 nM CF101 on the expression levels of PKAc, PKB/Akt, GSK-3β, β-catenin, cyclin D1 and c-Myc in HCT-116 cells. Serum-starved cells (for 18 h) were treated for 15 min with CF101 in the presence of 1% FBS. (**b**) To test the specificity of this response, the antagonist MRS 1523 (100 nM) was introduced to the culture system. Immunoblots showing the effect of CF101 on the cell growth-regulatory proteins in the presence and absence of MRS 1523 are depicted. (**c**) Immunoblots showing the effect of H89 (10 μM) and Worthmannin (100 nM) on the expression level of GSK-3β

tic mobility shift assay (EMSA) conducted with cell nuclei extracts revealed marked reduction in NF-κB DNA-binding capacity at 15, 30 and 60 min, suggesting a reduction in the NF-κB transcription activity at these time points (Figure 4b).



Discussion

In the present study, we followed the downstream signaling events taking place subsequent to A_3AR activation, resulting in tumor growth inhibition. These studies were conducted in a xenograft nude mice model, and were confirmed *in vitro*.

In mice treated chronically for 20 days with CF101, receptor protein downregulation was noted shortly after CF101 administration. Later on, prominent A_3AR

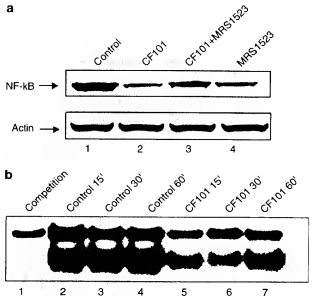


Figure 4 NF-κB expression level in cell lysates and EMSA in nuclear extracts. HCT-116 colon carcinoma cells were incubated for 15, 30 and 60 min at 37°C with 10 nM CF101. (**a**) WB analysis of whole-cell protein extracts conducted at 15 min of incubation in the absence and presence of the antagonist MRS 1523 (100 nM) and (**b**) EMSA of HCT-116 nuclear extracts at different time points

Figure 3 Increase in GSK-3β expression level and activity upon treatment of HCT-116 cells with CF101 leads to decreased β-catenin expression level. Cells were depleted from serum for 18 h and treated with vehicle (control) or with CF101 (10 nM or 10 μM) in the presence of 1% FBS for the times and concentrations indicated. (**a**) The expression of nonphosphorylated GSK-3β and phosphorylated GSK-3β (GSK-3β-P) was determined in cell protein extracts by WB analysis. (**b**) The ability of CF101 to inhibit the expression level of c-Myc was counteracted by SB216763, an inhibitor of GSK-3β (c). GSK-3β activity in HCT-116 colon carcinoma cells was incubated for 15 and 30 min at 37°C with 10 μM CF101. (**d**) HCT-116 cells were treated with CF101 (10 nM) for 15 and 30 min in the presence and absence of lithium chloride. The latter counteracted the decrease in β-catenin expression level, indicating that the response is GSK-3β mediated. (**e**) LEF-1 analysis in the nuclear extracts of HCT-116 cells treated with CF101, as detailed above, for 30 and 60 min

expression was noted, demonstrating that A_3AR was fully expressed in the tumor cells after chronic treatment with CF101. These fluctuations may be attributed to receptor internalization, degradation and re-synthesis, which occurs subsequent to receptor activation. These data are the first to show A_3AR expression *in vivo*, and support the notion that colon carcinoma cells do not develop 'resistance' or 'tolerance' to chronic treatment with a synthetic A_3AR agonist. Supporting the above is our recent publication demonstrating that, upon activation of B16-F10 melanoma cells with IB-MECA, A_3AR was internalized and sorted to the lysosome for degradation. Later on, the receptor was resynthesized and recycled to the cell surface (Madi *et al.*, 2003).

In the present study, receptor functionality was demonstrated by the modulation in the expression level of key signaling cell growth-regulatory proteins downstream to receptor activation. This included down-regulation of PKAc and PKB/Akt and upregulation of GSK-3β. Additionally, the protein expression levels of β-catenin, LEF-1 and the two oncogenes cyclin D1 and c-Myc were found to be decreased.

These results are in accordance with our previous studies, which showed decreased PKAc and PKB/Akt levels upon treatment of B16-F10 melanoma cells with IB-MECA (Fishman *et al.*, 2002b; Madi *et al.*, 2003). PKAc is the catalytic subunit of PKA, known to be activated subsequent to increase in cAMP level. Activation of A_3AR is known to decrease adenylyl cyclase activity and cAMP formation, resulting in a decline in PKAc level. PKB/Akt has recently been shown to be phosphorylated and thereby activated by PKAc (Fang *et al.*, 2000). The PI3K arm was reported to be upregulated upon A_3AR activation via the $\beta\gamma$-subunit (Schutle and Fredholm, 2002), leading to an increase in the phosphorylated form of PKB/Akt. Here, we show that, in colon carcinoma cells, downregulation of PKB/Akt takes place upon receptor activation, suggesting that in tumor cells modulation of the PKA arm is the dominant event, leading to the downregulation of PKB/Akt. PKAc and PKB/Akt utilize GSK-3β as a substrate and, upon phosphorylation, GSK-3β activity is inhibited. The latter has been widely implicated in cell homeostasis, by its ability to phosphorylate a broad range of substrates including β-catenin, a key component of the Wnt pathway (Ferkey and Kimelman, 2000). In normal cells, GSK-3β phosphorylates β-catenin, thereby inducing its ubiquitination and degradation by the proteosome system (Morin, 1999). However, in tumor cells, GSK-3β fails to phosphorylate β-catenin, leading to its accumulation in the cytoplasm. It then translocates to the nucleus, where it acts in concert with LEF-1 to induce the transcription of the cell cycle progression genes such as cyclin D1 and c-Myc (Kolligs *et al.*, 2002).

In previous studies, we showed that A_3AR activation induced downregulation of cyclin D1 and c-Myc in melanoma and prostate carcinoma cells, via deregulation of some Wnt signaling proteins (Fishman *et al.*, 2002b, 2003; Madi *et al.*, 2003). We thus assume that the decreased expression level of β-catenin is responsible for the diminished level of cyclin D1 and c-Myc.

In the present study, we examined the effect of CF101 on HCT-116 colon carcinoma cells, known to be mutated in the β-catenin gene (CTNNB1) (Lovig *et al.*, 2002). Mutations of CTNNB1 were found at the GSK-3β consensus phosphorylation site of β-catenin, that is, a deletion of serine 45 that occurs at a putative phosphorylation target of GSK-3β (Ilyas *et al.*, 1997). Surprisingly, we found that downregulation of β-catenin expression, which occurred upon CF101 treatment, was subsequent to an increase in the level of GSK-3β, notwithstanding the previously described, aforementioned mutation. Moreover, treatment of the cells with lithium, which directly inhibits the activity of GSK-3β, reversed the β-catenin level to that of the control. It thus seems that CF101 circumvents the inability of GSK-3β to phosphorylate β-catenin, leading to its susceptibility to degradation. Support for the involvement of β-catenin in the downregulation of cyclin D1 and c-Myc may be found in the data showing that nuclear level of LEF-1 was downregulated upon CF101 treatment. Furthermore, the GSK-3β inhibitor SB216763 counteracted the ability of CF101 to downregulate c-Myc, thus confirming that the events downstream to β-catenin are also mediated via GSK-3β.

An additional mechanism which may account for the downregulation of c-Myc and cyclin D1 is the direct phosphorylation of the two oncogenes by GSK-3β. It was recently shown that GSK-3β phosphorylates c-Myc at Thr-58 and cyclin D1 at Thr-286, thereby triggering their degradation (Alt *et al.*, 2000; Sears *et al.*, 2000).

The decreased level of PKB/Akt prompted us to examine the involvement of an additional important signaling protein, NF-κB, known to be phosphorylated and activated by PKB/Akt and additional downstream kinases. Since NF-κB is also involved in the transcription of cyclin D1 and c-Myc (Karin *et al.*, 2002), its decreased level may also attribute to the diminished expression of the two cell cycle genes.

The Wnt and the NF-κB signaling pathways are interconnected at the level of cyclin D1 and c-Myc. Both β-catenin and NF-κB control the transcription of these genes, thereby acting as a sensor for growth signals. Taken together, we propose here a model in which activation of the A_3AR induces modulation of PKAc and PKB, which on one hand upregulates GSK-3β, leading to phosphorylation and ubiquitination of β-catenin. On the other hand, remarkably, the similarity between the *in vitro* and *in vivo* data supports the notion that signaling proteins involved with the Wnt and NF-kB pathways are responsible for the observed modulation of cell growth-regulatory proteins.

The finding that cyclin D1 and c-Myc were down-regulated upon A_3AR activation both *in vitro* and *in vivo* is highly important in light of the bulk literature showing that most human cancers are characterized by overexpression of the two oncogenes (Hosokawa and Arnold, 1998; Parrella *et al.*, 2001; Masuda *et al.*, 2002). In some malignancies, overexpression of these proteins may serve as a marker of poor prognosis (Chana *et al.*,

2002; Nguyen *et al*., 2003). The importance of these two oncogenes in modulating the tumorigenic responsc was evidenced by the introduction of an antisense cyclin D1 or c-Myc sequence to malignant cells. This led to the inhibition of growth, the induction of apoptosis and the enhancement of sensitivity to chemotherapeutic agents (Van Waardenburg *et al*., 1997). Additionally, Jain *et al*. (2002) showed that brief MYC inactivation induced sustained loss of neoplastic phenotype.

Taken together, the molecular model that transpires upon activation of A_3AR with CF101 includes down-regulation of PKAc with a subsequent decrease in PKB/Akt expression level. This may lead on one hand to upregulation of the unphosphorylated form of GSK-3β and the phosphorylation and ubiquitination of β-catenin, resulting in the inhibition of translation of cyclin D1 and c-Myc. Additional events taking place downstream to PKB/Akt include decreased expression and DNA-binding capability of NF-κB, leading also to downregulation of cyclin D1 expression level.

The capability of CF101, a small orally bioavailable molecule, to downregulate cyclin D1 and c-Myc levels both *in vitro* and *in vivo* suggest that the compound is an attractive candidate to be developed as an anticancer agent.

Materials and methods

Reagents

CF101 is a GMP grade of the A_3AR agonist 1-deoxy-1-amino]-9*H*-purine-9-yl]-*N*-methyl-(-D-ribofuranuronamide) (IB-MECA), and was synthesized for Can-Fite BioPharma by Albany Molecular Research Inc., Albany, NY, USA. MRS 1523, a highly selective A_3AR antagonist, was purchased from RBI/Sigma (Natick, MA, USA). For both reagents, a stock solution of 10 mM was prepared in DMSO and further dilutions in RPMI medium were performed. Lithium chloride and H89 were purchased from Sigma Israel, and SB216763 was purchased from Biomol Research Laboratories Inc. (Plymouth, USA). RPMI, fetal bovine serum (FBS) and antibiotics for cell culturcs were obtained from Beit Haemek, Haifa, Israel.

Rabbit polyclonal antibodies against murine and human A3AR and the cell growth-regulatory proteins PKAc, PKB/Akt, c-Myc, GSK-3β, phosphor-specific GSK-3β (S9), β-catenin, cyclin D1 and LEF-1 and β-actin were purchased from Santa Cruz Biotechnology Inc., CA, USA.

Effect of CF101 on the growth of HCT-116 colon carcinoma in nude mice and assessment of A_3AR expression and cell growth-regulatory proteins in tumor lesions

Mice were maintained on a standardized pelleted diet and supplied with tap water. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Can-Fite BioPharma, Petah Tikva, Israel.

Nude malc Balb/*c* mice, aged 2 months, weighing an average of 20 g, were obtained from Harlan Laboratories, Jcrusalcm, Israel. HCT-116 colon carcinoma cells (1.2×10^6) were subcutaneously injected into the flank of the mice. When tumor reached 150 mm^3 in size, CF101 (10 μg/kg body weight) was administered orally twice daily for 20 days. The control group was treated orally twice daily with the vehicle only. Tumor size (width (W) and length (L)) was measured twice weekly with a caliber, and calculated according to the following formula: tumor size $= (W)^2 \times L/2$.

After 20 days of treatment and prior to terminating the study, the CF101-treated mice were divided into two groups. (A) mice treated for 20 days with CF101 and killed 16 h after last treatment; (B) mice treated for 20 days with CF101, received additional treatment on day 21 and killed 2 h later. Tumor lesions from the two groups and the control were then excised, homogenized (Polytron, Kinematica) and protein was extracted. WB analysis was carried out to determine the A_3AR expression level and additional cell growth-regulatory proteins. Each group contained 15 mice and the study was repeated three times. The results depicted are a representative experiment.

WB analysis

WB analysis of the following samples was carried out: (A) tumor lesions derived from CF101 and vehicle-treated nude mice inoculated with HCT-116 colon carcinoma cells (detailed above). (B) HCT-116 human colon carcinoma cells were serum starved overnight and then incubated with CF101 (10 nM or 10 μM) in the presence and absence of MRS 1523 (100 nM), H89 (10 μM), Worthmanin (100 nM), and/or SB216763 (1 μM) for time periods, as specified below, at 37°C. Samples were rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50 mM Tris buffer pH = 7.5, 150 mM NaCl, 0.5% NP-40). Cell debris were removed by centrifugation for 10 min, at 7500 *g*. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50 μg) were separated by SDS–PAGE, using 12% polyacrylamide gels. The resolved proteins were then electro-blotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with 1% BSA and incubated with the desired primary antibody (dilution 1:1000) for 24 h at 4°C. Blots were then washed and incubated with a secondary antibody for 1 h at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison, WI, USA). Data presented in the different figures are representative of at least four different experiments.

Preparation of nuclear extracts

Nuclear extract proteins from CF101-treated and control HCT-116 cells were prepared by incubating the cells for 15 min on ice in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.5 mM PMSF. Following incubation, Nonident P-40 (10%) was added, cells were vortexed for 10 s and centrifuged. The pellet was resuspended in a buffer containing 20 mM HEPES (pH = 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF, rocked on a shaker for 15 min at 4°C and centrifuged. Protein was quantified utilizing Bio-Rad protein assay dye reagent.

GSK-3β immunoprecipitation

HCT-116 human colon carcinoma cells were serum starved overnight and then incubated with CF101 (10 μM) for 30 min at 37°C. After isolating protein, 300 μg from each sample was removed for immunoprecipitation. The samples were cleared by incubating for 2 h with 1 μg/sample of rabbit IgG and 10 μl/sample of GammaBind Sepharose (Pharmacia, Piscataway, NJ, USA). After centrifuging, the supernatants were transferred to a tube containing 3 μg/sample of Ab against GSK-3β bound to GammaBind Sepharose, and then rotated at 4°C

overnight. The beads were subsequently washed three times with high salt buffer (1 M Tris-HCl pH 7.4, 0.50 M NaCl, and 1% Nonidet P-40) and three times with lysis buffer without protease inhibitors. The immunoprecipitated complexes were used in a kinase activity assay.

GSK-3β activity assay

After immunoprecipitating GSK-3β from HCT-116 cells, the protein-containing pellet was washed twice with kinase buffer (20 mM $MgCl_2$, 25 mM HEPES, 20 mM glycerophosphate, 20 mM *p*-nitrophenylphosphate, 20 mM sodium orthovanadate and 2 mM DTT). The pellet was then suspended in 20 μl kinase buffer and the following ingredients were added: 20 μM ATP, 5 μCi ATP (BLU 002Z; DuPont-NEN, Boston, MA, USA) and 10 μg myelin basic protein (MBP; Sigma). The total volume of sample plus additions at this point was 25 μl. The reaction was continued for 30 min at 25°C and then stopped by the addition of 25 μl/sample of 2 × sample buffer. The samples were boiled for 5 min, then run on a 12% SDS–PAGE gel. The gel was dried, and autoradiography performed to visualize the ^{32}P-labeled MBP.

EMSA of NF-κB

To carry out the gel shift assay, double-stranded oligonucleotides for the consensus sequence of NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were end-labeled with ^{32}ATP (Amersham) using polynucleotide kinase (Promega). Nuclear protein extracts (3 μg) were incubated for 30 min at room temperature with the end-labeled DNA (1 μg) in binding buffer containing 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM DTT, 25 mM EDTA, 20% glycerol, 50 mM Tris-HCl pH 7.5 and 2 μg/sample of poly (dI-dC), in a final volume of 25 μl. Competition with unlabeled oligonucleotide of NF-κB binding sequence at a 100-fold molar excess was used to analyse specific bands. The reaction product was analysed by 6% nondenaturating polyacrylamide gel electrophoresis. The specific bands were visualized by X-ray autoradiography.

Statistical analysis

The results were evaluated using the Student's *t*-test, with statistical significance set at $P<0.05$. Comparison between the mean values of different experiments was carried out.

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